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Sex differences in oxidative stress responses of tropical topshells (*Trochus histrio*) to increased temperature and high pCO_2

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ABSTRACT

Given scarcity of knowledge on gender ecophysiological responses of tropical marine organisms to global climate change, the major aim of this research was to investigate potential sex differences in oxidative status of topshell *Trochus histrio*, after a combined exposure to increased temperature and pCO_2. Lipid peroxidation, heat-shock response and antioxidant enzymatic activities were evaluated. Lipid peroxidation varied differently between sexes, with males undergoing cellular damage under high pCO_2, which was elevated temperature-counteracted. Heat shock response was thermo- and sex-regulated, with males exhibiting significantly higher heat shock proteins production than females. Catalase activity increased with temperature and was exacerbated in combination with hypercapnia, being highest in females, while glutathione S-transferases activity peaked in males. These results clearly support the existence of distinct physiological strategies to cope oxidative stress between sexes, apparently more efficient in females, and also reinforce for the need of encompassing sex as meaningful variable in future biomarker studies.

1. Introduction

The increase in atmospheric carbon dioxide (CO_2) levels has been anticipated as the primary cause of ocean acidification, which is now considered one of the most pervasive human impacts on global marine biodiversity. The absorption of increasingly higher CO_2 concentrations into the ocean translates into a decline in the mean ocean pH, predicted to vary between 0.13 and 0.42 units by the end of the century (IPCC, 2014). In parallel with this phenomenon a substantial increase in the global average temperature (0.3–4.8 °C) is predicted to occur until 2100 (IPCC, 2014). Ocean warming and acidification have severe impacts for calcifying marine organisms, particularly molluscs, the major producers of calcium carbonate. Recent meta-analyses have identified shelled molluscs as one of the most vulnerable invertebrate taxa under changing ocean conditions (Byrne and Przeslawski, 2013; Kroeker et al., 2013; Wittmann and Pörtner, 2013), thereby becoming prime models for climate change experimentation (Parker et al., 2013).

Climate and toxic-related environmental changes can often disrupt metabolic homeostasis of the organisms. Such disruption can occur through direct mechanisms including temperature effects on enzyme properties, protein conformation, and lipid fluidity of biological membranes, as well as through indirect pathways, such as the overproduction of reactive oxygen species [ROS; e.g. superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-)] (Santoro and Thiele, 1997; Pannunzio and Storey, 1998). These radicals can damage DNA, proteins and lipids and thereby endanger cellular and organism fitness and functions, compelling them to invest more energy into cell repair (Pöhlmann et al., 2011). Peroxidation, the most common cellular damage caused by oxidative stress, is the reaction of ROS with lipids, constituents of cell membranes (Lesser, 2011), resulting in malondialdehyde (MDA) production, among other reactive end-products (Uchiyama and Mihara, 1978; Pannunzio and Storey, 1998; Lopes et al., 2013). Aerobic organisms have developed cytoprotective mechanisms including antioxidant enzymes to cope with hostile environmental conditions (Lopes et al., 2013; Troschinski et al., 2014). These biomarkers have been widely used in several studies proving to be valuable tools to understand biochemical responses of the organisms to both environmental contamination by anthropogenic pollutants (Sampaio et al., 2018) or climate related-changes (Lopes et al., 2013; Matoo et al., 2013; Rosa et al., 2014) and ultimately to evaluate ecosystems' health. Antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) are critically important to minimize detrimental effects caused by ROS produced in cells under physiological strain (Abele and Puntarulo, 2004; Lopes et al., 2013). Glutathione S-

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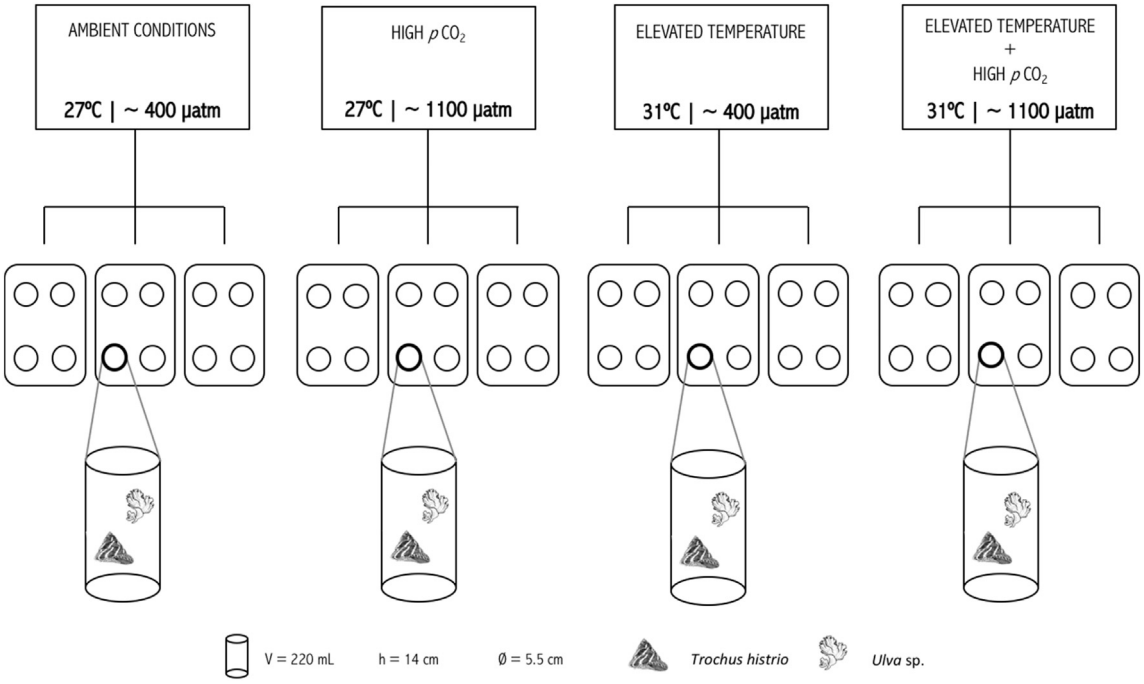

Fig. 1. Schematics of experimental design. Main treatments: ambient condition, high pCO_2, elevated temperature, high temperature and pCO_2.

transferases (GSTs) play an essential role in the transformation of xenobiotics into other conjugates as part of a detoxification pathway (Lesser, 2011; Lopes et al., 2013).

Moreover, marine organisms also produce chaperones (heat shock proteins, HSP), in order to stabilize and restore denatured proteins and thereby preventing formation of cytotoxic aggregates (Hartl, 1996; Fink, 1999). The thresholds of heat shock response in marine organisms are correlated with habitat temperature and the stress levels at which the organisms are usually exposed to (Feder and Hofmann, 1999). Considering that tropical species already live closer to their thermal tolerance limit (Rosa et al., 2014), the latest official reports (IPCC, 2014) highlight for their high vulnerability facing this rate of ongoing changes.

Topshells or banded *Trochus* spp. snails live on intertidal and shallow subtidal in the tropical Indo-West Pacific coral reefs (Lee and Lynch, 1997). They are nocturnal grazing herbivores and detritivores (Jolivet et al., 2015) and are among the most economically valuable marine snails in the tropical Pacific, constituting a highly consumed traditional food and an important leading export item to Asia and Europe as their aragonite shells are primary raw material for mother-of-pearl buttons. Each year, 3000–6000 tons of *Trochus* spp. are estimated to be harvested for subsistence and commercial purposes (Shokita et al., 1991; Ramakrishna and Sivaperuman, 2010). Nevertheless, their ecophysiology remains poorly understood and in the face of upcoming global changes, it becomes crucial to understand how increasing ocean temperature and high pCO_2 may or may not affect the biochemistry of *Trochus histrio*.

Whilst the ecophysiological impacts of medium and long-term changes in temperature and oceanic uptake of atmospheric CO_2 have been increasingly studied, the gender ecophysiological effects on oxidative status of tropical marine organisms have received much less attention. Some studies have demonstrated that response to thermal shifts is influenced by sex and commonly attributed to differences in the physiology and energy allocations between males and females (Madeira et al., 2012; Bedulina et al., 2017). Taking this evidence into consideration, the main goal of the present work was to evaluate, for the first time, the short-term effects of combined thermal and hypercapnia stress on the biochemical responses of different *T. histrio* genders. Can different sexes have distinct strategies to cope with the same climate stressors? This is the main question addressed along the manuscript. For that purpose, we performed a 15-day experiment to investigate potential sex differences in lipid peroxidation, heat shock response, and antioxidant enzymes (e.g. CAT and GSTs) machinery in economically relevant topshell *T. histrio* under a realistic scenario of increased temperature ($\Delta T = 4\,^{\circ}C$) and high pCO_2 ($\Delta pCO_2 \approx 700$ µatm).

2. Materials and methods

2.1. Organism collection and laboratory acclimation

Topshells (*T. histrio*) were collected during low tide by local fishermen from the Indo-West Pacific region, near Bali (Indonesia) coastline, during summer (July 2015). The transport to aquaculture facilities of Laboratório Marítimo da Guia (Cascais, Portugal) was ensured by the Tropical Marine Centre UK, a marine aquarium wholesaler recognized for its efforts on the sustainable fishing of reef organisms and promotion of animal welfare. Topshells were 1-week laboratory acclimated under seawater conditions mimicking those at collection site: salinity = 35 ± 0.93 (V2 refractometer, TMC Iberia, Portugal); water temperature = 27 ± 0.81 °C (TFX 430 Precision Thermometer, WTW GmbH, Germany) and pH = 8.0 ± 0.2 / $pCO_2 \sim 400$ µatm (SG8 – SevenGo pro™ pH/Ion meter, Mettler-Toledo International Inc., Switzerland). During initial acclimation to laboratory conditions, topshells were randomly placed in twelve 50 L tanks, each part of individual recirculating aquaculture systems (RAS) with mechanical and biological filtration (bio-balls and skimmers), as well as additional UV disinfection. During laboratory acclimation and exposure to climate change conditions, topshells were fed ad libitum with green algae *Ulva* sp. from the same site of animals' collection.

After the acclimation period, temperature and pCO_2 were gradually regulated to mimic scenarios of ocean warming ($\Delta T = +\ 4\,^{\circ}C$) and acidification ($\Delta pH = -0.4$ units, i.e. $\Delta pCO_2 \sim +\ 700$ µatm) predicted for the end of the century (IPCC, 2014), in a full factorial design. In more detail, and before starting the 15 day-exposure period, the pH, as a proxy of pCO_2, was slowly decreased, at a rate of 0.1 units per day until reaching the desired pH (from 8.0 to 7.6). In parallel, temperature

was gradually increased up to 31 °C, at a rate of 1 °C per day. These procedures of gradual lowered pH and increased temperature were performed in order to minimize stress imposed to the organisms and lasted 4 days. Thus, the laboratory acclimation lasted a total of 11 days (7 + 4) before the exposure experiment started.

2.2. Experimental set-up

The experimental design is presented in Fig. 1. Topshells were exposed for 15 days to 4 distinct treatments: 1) ambient conditions (27 °C – mean sea surface temperature at collection site, pCO_2 ~ 400 μatm), 2) high pCO_2 (27 °C, pCO_2 ~ 1100 μatm), 3) high temperature (31 °C – future sea surface temperature for 2100 (+4 °C), pCO_2 ~ 400 μatm) and 4) high temperature and pCO_2 (31 °C, pCO_2 ~ 1100 μatm). Future scenarios of altered temperature and pH were set following IPCC's Representative Concentration Pathways (RCP) scenario 8.5 (IPCC, 2014) and within the range of nearshore temperatures observed in close areas to the natural habitat of topshells (Pearce and Feng, 2013). Indeed, for several consecutive days, high ocean temperatures were recorded during the austral summer along the Indo-West region, where topshells inhabit, with peak nearshore temperatures rising to ~ 5 °C above the average (Pearce and Feng, 2013).

The experiment was performed in twelve recirculating seawater systems (three per treatment, 55 L each), as presented in Fig. 1. Each system included 4-drilled (to allow water circulation inside them) cylindrical plastic containers (5.5 cm diameter × 14 cm height / 220 mL), each one with a topshell ad libitum fed with green algae. Thus, a total of 12 individuals (4 topshells × 3 replicates) were present for each experimental treatment. To avoid animals escaping, a transparent acrylic plate covered each system. Considering the intertidal to shallow subtidal life habit of topshells (Lee and Lynch, 1997) we guaranteed they were not permanently submerged during the experiment. The cylindrical containers where the animals were placed were not full of seawater, existing a 5 cm air-filled space to give animals the possibility to be emerged whenever they wanted.

Flow-through aquatic systems were set in order to maintain total alkalinity (A_T), total inorganic carbon (C_T) speciation due to bacterial activity, and acidification of treatments. Experimental tanks were set in a completely randomized outline, following procedures described in Repolho et al. (2017). Natural seawater was pumped directly from the ocean into a 5 m^3 storage tank, UV-sterilized (Vecton 600, TMC Iberia, Portugal) and 0.35 μm filtered (Harmsco, Florida, USA) before being transferred to mixing (n = 12) and respective experimental tanks (n = 12). Photoperiod was fixed to 12 h light: 12 h dark. Nitrate cycle components (e.g. ammonia and nitrite), together with temperature, pH and salinity (V2 refractometer, TMC Iberia, Portugal) were measured daily. Temperature was increased with 200 W submerged heaters. The pH values, as proxy measurement, were adjusted automatically by a controller system (Profilux 3.1, GHL, Germany) connected to pH probes for each replicate tank. Monitoring of pH was performed every 2 s interval, with pH values being lowered through the injection of certified CO_2 gas (Air Liquide, Portugal) or upregulated by aerating the tanks. Quantification of pH was determined using a pH meter (826 pH mobile, Metrohm, Germany) connected to a glass electrode (Schott IoLine, SI analytics, ± 0.001), calibrated with TRIS-HCl (TRIS) and 2-aminopyridine-HCl (AMP) (Mare, Belgium) seawater buffers. Recording of pH values was performed under temperature-controlled conditions using a water bath (± 0.1 °C, Lauda, Germany). Specifics of seawater carbonate system (pCO_2, HCO_3^-, CO_3^{2-}, C_T, aragonite saturation state) are summarized in Supplementary Table S1 and were calculated once every week (total n = 10 per experimental treatment) from A_T (Sarazin et al., 1999) using the CO2SYS software (Lewis and Wallace, 1998), set to dissociation constants (Mehrbach et al., 1973) as optimized by Dickson (2010).

2.3. Biochemical analyses

2.3.1. Processing, sex distinction and preparation of tissue extracts

At the end of the experiment and after shell removing, soft tissues of topshells were weighted. Based on previous studies (Lopes et al., 2013; Matoo et al., 2013; Rosa et al., 2014; Sampaio et al., 2018), lipid peroxidation (via MDA quantification) and HSPs, coupled with commonly quantified antioxidant enzymes (e.g. CAT and GSTs) were selected to investigate their usefulness to be used as efficient biomarkers to assess potential sex differences under a climate change scenario. Supported by evidence reported in the previous studies, we selected these biomarkers as the most representative and that could provide a better response to the studied climate change-related stressors. Determination of sex was only possible after death and was mostly based in visual characteristics: the male gonads were whitish and creamy whilst the female ones were of green-brownish aspect, similarly to described for *Tectus niloticus* (formerly *Trochus niloticus*) (Hahn, 1993; Ramakrishna and Sivaperuman, 2010). Based on the literature available (Hahn, 1993) we may assume that *T. histrio* males and females evidenced no differences in energy allocation due to reproductive status, since they were both juveniles (2–3 cm diameter) (Hahn, 1993) and still sexually immature. At the beginning of the experiment, 12 individuals were distributed for each experimental treatment, however as we did not know the exact number of males and females until they were dissected, we only used 10 organisms per treatment (5 per sex) for analysis - the same sampling size used by Bedulina et al. (2017).

After dissection, soft tissues were homogenized by using a glass/PTFE Potter Elvehjem tissue grinder in a phosphate buffered saline solution (PBS) and then centrifuged for 20 min at 14000 × g and 4 °C. Subsequent enzyme results were normalized with total protein content following Bradford (1976).

2.3.2. Determination of malondialdehyde concentration and heat shock response

Degree of lipid peroxidation was determined by quantifying the end-product malondialdehyde (MDA). As in previous studies (for detailed MDA protocols description please see Rosa et al., 2014) we used the thiobarbituric acid reactive substances (TBARS) assay according to Uchiyama and Mihara (1978) and adapted to the microplate reader. Briefly, 12.5 μL of sodium dodecyl sulfate (8.1%), 93.5 μL of trichloroacetic acid (TCA 20%, pH = 3.5) and 93.5 μL of thiobarbituric acid (TBA 1%) were added to 10 μL of each homogenate. TCA precipitation is a common method frequently used to remove interfering compounds. TBARS react with MDA, increasing sample fluorescence according to MDA concentration. Spectrophotometric measurements were referenced within a malondialdehyde (dimethylacetal, Merck, Switzerland) standard eight-point calibration curve (0–0.3 μM TBARS) and results were expressed relatively to sample total protein (pmol mg^{-1} total protein).

Heat shock response (Hsp70/Hsc70) was assessed through Enzyme-Linked Immunosorbent Assay (ELISA), by adapting a protocol from Njemini et al. (2005) to a microplate reading. In essence, homogenates (20 μL) were diluted in 980 μL phosphate buffer (PBS). Subsequently, 100 μL of each dilution was added to a microplate and incubated overnight at 4 °C. Microplates were washed (PBS with 0.05% Tween-20) and incubated with a primary antibody anti-Hsp70/Hsc70 (Sigma-Aldrich, Germany), non-binding antibodies were removed and a secondary antibody (anti-mouse IgG Fab specific, 1 μg mL^{-1} ALP conjugate from Sigma-Aldrich, Germany) was added. Finally, 100 μL substrate *p*-nitrophenyl phosphate tablets (Sigma-Aldrich, Germany) and 50 μL stop solution (3 M NaOH) were added with a 30 min interval in between. Hsp70/Hsc70 quantification was obtained from an absorbance/concentration calibration curve based on purified Hsp70 active protein (Acris) serial dilutions (from 0 to 2000 μg mL^{-1}) and results expressed relatively to sample total protein (μg mg^{-1} total protein).

2.3.3. Antioxidant enzymes

Catalase activity was assessed by adapting the method described by Johansson and Borg (1988). To the homogenate were added 100 µL of 100 mM potassium phosphate, 30 µL of methanol, 30 µL of potassium hydroxide (10 M KOH), 30 µL of purpald (34.2 mM in 0.5 M HCl) and lastly 10 µL of potassium per iodate (65.2 mM in 0.5 M KOH). Using a microplate reader (Asys UVM 340, Biochrom, USA), enzymatic activity was determined spectrophotometrically at 540 nm. The results expressed in relation to the total protein content (nmol min^{-1} mg^{-1} protein).

Activity of glutathione S-transferases (GSTs) was based on the procedure described by Habig et al. (1974) and adapted for 96-well microplate (e.g. Rosa et al., 2014). Shortly, the conjugation of 1-Chloro-2,4-dinitrobenzene (CDNB) with the thiol group of glutathione increases absorbance allowing for enzymatic activity to be determined spectrophotometrically at 340 nm absorbance. Throughout this assay the homogenate is incubated with 200 mM L-glutathione (reduced), 100 mM of CDNB solution and Dulbecco's PBS. Results were expressed relatively to the total protein of the sample (nmol min^{-1} mg^{-1} total protein). For further details about antioxidant enzymes protocols please see Lopes et al. (2013) and Rosa et al. (2014).

2.4. Statistical analyses

Generalized Linear Models (GLM) were applied to detect significant differences among factors. As previous analyses revealed no significant differences among tanks pertaining to the same temperature and/or pCO_2 treatment (GLM analysis, $p > 0.05$ for all treatments), replicates were pooled and $n = 5$ for each sex and *per* experimental treatment was considered. Model residuals were checked for homogeneity of variances, independence and leverage to validate final models. For some physiological endpoints (e.g. Hsp70 and CAT) data was fitted using Gaussian family. Whenever normality assumptions were violated and taking into consideration that data transformation masks distance between data points and therefore affects statistical validity (most evident in the presence of interactions between factors) (Zuur et al., 2010), we resorted to more suitable distribution fitting, through the use of gamma models (e.g. MDA and GSTs). Temperature (2 levels: 27 °C, 31 °C), CO_2 (2 levels: ~400 µatm, ~1100 µatm) and Sex (2 levels: Male, Female) were used as explanatory variables or factors. The best model for each endpoint was ultimately chosen through the lowest Akaike Information Criterion (AIC) value, a widespread indicator that balances model complexity with model quality of fitness (Quinn and Keough, 2002). Moreover, it is important to note that all information plotted in the figures in the results section has already been deemed significant by the AIC-chosen GLM models, i.e. all the factors presented in the graphs were significant. Tukey *post-hoc* tests were applied to better scrutinize the effect of explanatory variables on each measured endpoint. All statistical analysis were performed on R Studio (R Development Core Team, 2016).

3. Results

3.1. Lipid peroxidation and heat shock response

No mortalities were registered during the exposure period for all different treatments. MDA production was modelled by a triple interaction among temperature, pCO_2 and sex ($p = 0.023$, GLM analysis in Table 1A, Fig. 2A). In more detail, lipid peroxidation was significantly induced by exposure to increased pCO_2 in males, however, when combined with high temperature such effect was counter-balanced and cellular damage risk lowered. Levels of MDA in females were not affected by either temperature or pCO_2 and no cellular damage was perceived.

The variables that significantly affected heat shock response were the temperature ($p < 0.001$, Fig. 2B, GLM analysis in Table 1B) and the sex ($p = 0.019$, Fig. 1C, GLM analysis in Table 1). The production of heat shock proteins was significantly stimulated under increased temperature, with males evidencing significantly higher values than females (Fig. 1B,C). No interactions among variables were found ($p > 0.05$, GLM analysis in Table 1B).

Table 1
GLM analysis of lipid peroxidation and consequent MDA (malondialdehyde) build-up and heat shock protein response in *T. histrio* under crossed treatments of temperature (T, 2 levels: 27 °C and 31 °C), pCO_2 (CO_2, 2 levels: 400 µatm and 1100 µatm) and sex (Sex, 2 levels: male and female). Model formula on top, family and respective model AIC in the bottom. Bold values indicate $p < 0.05$.

	Estimate	Std error	t value	Pr(> |t|)
A				
Malondialdehyde quantification				
*GLM: MDA in function of T * CO2 * Sex*				
(*Intercept*)	3.437	0.662	5.190	< **0.001**
T	0.184	0.962	0.191	0.850
CO_2	0.228	0.968	0.236	0.815
Sex	−2.227	0.702	−3.172	**0.003**
*T * CO_2*	2.276	1.678	1.357	0.184
*T * Sex*	2.047	1.191	1.718	0.095
*CO_2 * Sex*	3.406	1.365	2.496	**0.018**
*T * CO_2 * Sex*	−5.234	2.193	−2.387	**0.023**
Family = Gamma (inverse)			AIC = −50.057	
B				
Heat shock protein response				
GLM: Hsp70 in function of T + Sex				
(*Intercept*)	292.130	55.860	5.230	< **0.001**
T	328.910	64.500	5.099	< **0.001**
Sex	158.800	64.500	2.462	**0.019**
Family = Gaussian			AIC = 543.83	

3.2. Antioxidant enzymes

Regarding the antioxidant enzymatic machinery, CAT activity was modelled by the interaction of temperature with pCO_2 (T × pCO_2, $p = 0.022$, GLM analysis in Table 2A, Fig. 3A) and also by a strong interaction between temperature and sex ($p = 0.007$, GLM analysis in Table 2A, Fig. 3B). In more detail, and by comparison with ambient conditions, CAT activity was significantly enhanced under single warming in females and additively exacerbated combined with high pCO_2 (Fig. 3B). Elevated temperature, as single stressor, did not produce any significant alterations in CAT activity in males, suggesting lower risk of oxidative stress.

Significant interactions in GSTs activity were registered between temperature and pCO_2 (T x pCO_2, $p = 0.015$, GLM analysis) and temperature and sex (T x CO_2, $p < 0.001$, GLM analysis), as presented in Table 2B, Fig. 3C,D. In more detail, under an isolated scenario of high pCO_2, GSTs activity was slightly depressed, which was counter-balanced by single warming and also when both stressors are combined (Fig. 3C). Higher temperature stimulated GSTs activity for both sexes, most evident in males (Fig. 3D).

4. Discussion

The present study demonstrated that short-term exposure to increased temperature and high pCO_2 produced sublethal effects on *T. histrio* physiology, as supported by the activation of cellular protective mechanisms and translated into changes in lipid peroxidation, heat shock response and antioxidant enzymatic machinery. Concerning snails, there are several studies using lipid peroxidation and antioxidant enzymes as efficient biomarkers of environmental contamination (Bhagat et al., 2016), anoxia (Pannunzio and Storey, 1998), and temperature and salinity fluctuations (Deschaseaux et al., 2011). However, to our knowledge, no previous studies have yet integrated the gender as a meaningful variable to investigate the interactive effects of high

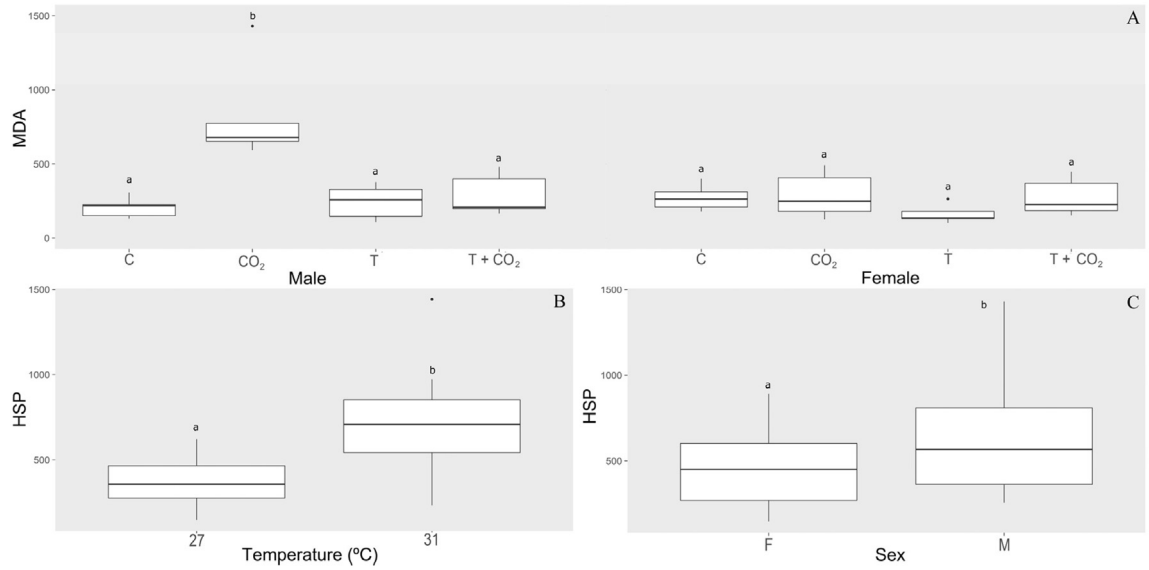

Fig. 2. Lipid peroxidation and heat shock response of *T. histrio* gonadal tissue and digestive gland ($n = 5$ *per* sex and experimental treatment). (A) Schematic boxplots (mean, box as 50% of values, plus confidence intervals showing 95% value range) presenting malondialdehyde (MDA) concentrations (expressed as pmol·mg^{-1} total protein) driven by a triple interaction between temperature (27 °C and 31 °C), pCO_2 (400 µatm and 1100 µatm) and sex (male and female). (B, C) Schematic boxplots (mean, box as 50% of values, plus confidence intervals showing 95% value range) presenting heat shock protein (Hsp70) concentrations (expressed as µg·mg^{-1} total protein) in function of temperature (27 °C and 31 °C) and sex (male and female) Graphs were plotted according to significant factors yielded by GLM analysis described in Table 1. C – ambient condition, CO_2 – high pCO_2, T – high temperature, T + CO_2 –high temperature and pCO_2. M – male, F – female. Different letters indicate significant differences among groups ($p < 0.05$, Tukey test).

Table 2
GLM analysis of catalase (CAT) and gluthathione S-transferases (GSTs) activities in *T. histrio* under crossed treatments of temperature (T, 2 levels: 27 °C and 31 °C), pCO_2 (CO_2, 2 levels: 400 µatm and 1100 µatm) and sex (Sex, 2 levels: male and female). Model formula on top, family and respective model AIC in the bottom. Bold values indicate $p < 0.05$.

	Estimate	Std error	t value	Pr(> |t|)
A				
Catalase activity				
*GLM: CAT in function of T * CO2 + T * Sex*				
(*Intercept*)	59.59	14.079	4.233	< **0.001**
T	137.45	19.910	6.904	< **0.001**
CO_2	−38.37	16.257	−2.360	**0.024**
Sex	2.906	16.257	0.179	0.859
*T * CO_2*	−55.00	22.990	−2.392	**0.022**
*T * Sex*	−66.22	22.990	−2.881	**0.007**
Family = Gaussian			AIC = 408.47	
B				
Glutathione S-transferases activity				
GLM: GST in function of T + Sex				
(*Intercept*)	0.017	0.002	7.221	< **0.001**
T	−0.013	0.002	−5.301	< **0.001**
CO_2	−0.002	0.001	−1.695	0.099
Sex	−0.012	0.002	−5.292	< **0.001**
*T * CO_2*	0.003	0.001	2.570	**0.015**
*T * Sex*	0.009	0.002	3.775	< **0.001**
Family = Gamma (inverse)			AIC = 490.82	

temperature and pCO_2 on oxidative stress endpoints. There is only one study, so far, focusing on the evaluation of the oxidative status of a temperate snail under such climate change context – the periwinkle *Littorina obtusata* (Cardoso et al., 2017) – but it ignores gender-based differences. Contrarily to the temperate periwinkle, which response was characterized by a general decline in the antioxidant defense (i.e. more pronouncedly GSTs and in a minor scale Hsp70, whilst CAT and MDA levels remained unaltered), tropical topshells showed to be quite resilient by enhancing their antioxidant protective mechanisms (i.e. specific chaperones and the antioxidant machinery) in a future scenario of climate change. This is also reinforced looking for survival and fitness trends. A 15-day exposure to warming (+4 °C) resulted in very high mortality rates (> 90%) and fitness decline in the temperate periwinkle *L. obtusata* (Cardoso et al., 2017), contrasting to the findings for *T. histrio* survival under the same duration exposure and temperature range.

In the literature there are some studies highlighting for strong evidence that elevated temperature, as single stressor, induces lipid peroxidation and heat shock protein synthesis in rocky intertidal (Deschaseaux et al., 2011), terrestrial (Troschinski et al., 2014; Dieterich et al., 2015) and freshwater snails (Axenov-Gribanov et al., 2015), which partially corroborates our results. The present findings revealed that elevated temperature individually did not cause differences in lipid peroxidation between sexes, however, under single hypercapnia, cellular damage occurred exclusively in males (without producing lethal effects), manifested by significantly higher MDA production. When both stressors were combined, MDA declined to background levels in males, indicative that single hypercapnia-deleterious effects were dampened by co-occurring elevated temperature, contributing for cellular damage lowering risk. Gender-based differences in lipid peroxidation suggest that *T. histrio* females may have developed alternative and more efficient physiological mechanisms (to be further investigated) than males, in order to minimize deleterious actions of oxygen-derived radicals in lipids' membranes imposed by single hypercapnia. It is known that lipid levels in the body constituents of marine invertebrates may vary considerably, with the physical environment, the dietary requirements of the organisms and the annual reproductive cycle being the most likely sources of fluctuation (Brazão et al., 2003). The fatty acids profiles of the gonads and soft tissues of two species of limpets (*Patella depressa* and *Patella ulyssiponensis*) revealed significant differences between sexes (Brazão et al., 2003), which may explain the findings of our study. Limpet males showed significantly higher percentages of PUFA (polyunsaturated fatty acids) from the (n-3) and (n-6) series [arachidonic acid (AA) and eicosapentaenoic acid (EPA)] and females significantly higher proportions of monounsaturated fatty acids (MUFA). Brazão et al. (2003) attributed such differences probably with the limpet's diet and position on the shore (which will ultimately affect their diet).

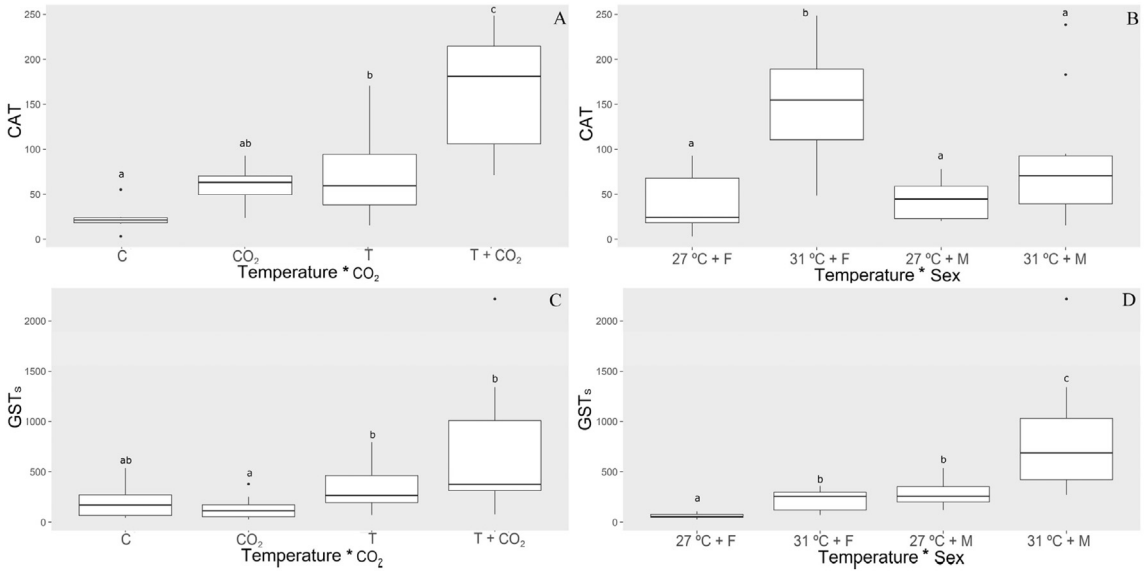

Fig. 3. Antioxidant enzymes responses of *T. histrio* gonadal tissue and digestive gland (n = 5 *per* sex and experimental treatment). (A, B) Schematic boxplots (mean, box as 50% of values, plus confidence intervals showing 95% value range) presenting catalase (CAT) concentrations (expressed as nmol.min^{-1}.mg^{-1} total protein) driven by a double interaction between temperature (27 °C and 31 °C) and pCO_2 (400 µatm and 1100 µatm) and temperature and sex (male and female). (C, D) Schematic boxplots (mean, box as 50% of values, plus confidence intervals showing 95% value range) presenting glutathione S-transferases (GSTs) concentrations (expressed as nmol·min^{-1}·mg^{-1} total protein) driven by an interaction between temperature (27 °C and 31 °C) and sex (male and female) Graphs were plotted according to significant factors yielded by GLM analysis described in Table 2. C – ambient condition, CO_2 – high pCO_2, T – high temperature, T + CO_2 –high temperature and pCO_2. M – male, F – female. Different letters indicate significant differences among groups ($p < 0.05$, Tukey test).

From a biochemical perspective, facing an impairment or excessive ROS production, higher than the antioxidant capacity response of the organism, the H_2O_2 is converted into hydroxyl ions (OH^-) capable of damaging proteins, lipids and DNA (Santoro and Thiele, 1997; Pannunzio and Storey, 1998). The hydroxyl ions react with PUFAs, taking away a H+ from the methylene group and starting the peroxidation, which cause the formation of a diene, leaving an unpaired electron on the carbon. Afterwards the fatty acid combines with O2 yielding highly reactive peroxyl radicals, resulting in the production of harmful lipid peroxidation by-products, such as MDA and 4-HNE (4-hydroxynonenal) (Shah et al., 2014). Based on this evidence, we hypothesized that higher MDA production in males was indicative of high availability of OH^- that were interacting with fatty acids carbon chains, resulting in several by-products, which may not occur in females under single hypercapnia.

Regarding heat shock response, and as reported recently by Bedulina et al. (2017) for the amphipod *Eulimnogammarus verrucosus*, significant differences in heat shock response were observed between sexes. Contrasting with amphipods, *T. histrio* males showed to be more sensitive face to a thermal stimulus than females, which was translated into higher production of Hsp70. Differences in the physiology and energy allocations between males and females may explain these deviations (Madeira et al., 2012; Bedulina et al., 2017). Under hostile environmental conditions, the induction of HSPs is recognized as a common and efficient survival strategy, protecting cells and tissues from non-optimal temperatures, thereby maintaining protein homeostasis. It has been suggested that expression of HSPs may reflect a physiological strategy of intertidal organisms to occupy ecological niches close to their thermal limits (Tomanek, 2010), as it can be applied to topshells. Biosynthesis of HSPs is known to be a costly energetic process, particularly under stressful conditions. Given that, Troschinski et al. (2014) argued that, up to a certain heat stress level, terrestrial snails (e.g. *Xeropicta derbentina*) choose to spend energy in the biosynthesis of enzymatic antioxidants, instead of Hsp70, which would explain high CAT activity associated with low levels of lipid peroxides and also decreased Hsp70 levels in terrestrial snails. However, our data do not entirely support this hypothesis, as the levels of HSPs and antioxidant enzymes (e.g. CAT and GSTs) remained generally high for both sexes, whilst lipid peroxides kept low and relatively stable (i.e. except for males under high pCO_2). Differences in the habitat type (terrestrial versus marine), survival strategies, tolerance to environmental stressors (desiccation and aestivation in land versus tides, temperature, pH and salinity fluctuations in the ocean), feeding ecology, sex, and physiological and metabolic processes, may explain distinct oxidative stress responses between terrestrial and marine snails.

Findings of Axenov-Gribanov et al. (2015) for the freshwater gastropod *Lymnaea stagnalis* support the results obtained for *T. histrio* by revealing that increments in temperature resulted in the activation of HSPs and overall enhancement of GSTs activity. This indicates that GSTs were involved in the effective elimination of the secondary products of lipid peroxidation, including conjugated dienes and trienes (Fridovich, 1998), as evident in males. We suggest that high levels of CAT and GSTs reinforce the central role of these enzymes as effective barriers against oxidative stress in topshells, providing them permanent protection against the cytotoxic action of ROS (Troschinski et al., 2014) and contributing for thermotolerance of these organisms. In contradiction with aforementioned studies no signs of impaired cellular redox status were observed after 15-week-exposure of clams and oysters to warming (+5 °C) and hypercapnia (~800 µatm CO_2), although high mortality rates under single warming, ~44% and 95%, respectively (Matoo et al., 2013). These reports emphasize how oxidative stress responses can be highly variable and species-specific (Pannunzio and Storey, 1998; Pöhlmann et al., 2011). In this context, our study adds new insights to this field of knowledge by revealing that oxidative stress responses are also sex-specific. The strong significance and interactions found among variables (temperature, CO_2 and sex) for the battery of biochemical endpoints analyzed reinforce their relevance as efficient tools to investigate oxidative stress in organisms under a changing ocean. The present results clearly support the existence of different physiological strategies to cope oxidative stress between sexes: for females there was a higher prevalence of CAT activity, whilst for males, GSTs and production of Hsp70 dominated the antioxidant and chaperones protective response. As catalase is an antioxidant enzyme directly linked with removal of ROS ions, its high activity in females

suggest an effective prevention against MDA production, contrarily to the observed in males. Thus, preventing directly ROS formation, in opposition of refolding proteins and eliminate MDA, appears to be a more successful strategy, enabling females to better cope with oxidative stress than males, particularly under an acidified scenario. If the exposure period would be longer, based on the evidence found in the present study, we might argue that survival of *T. histrio* would not be affected, although some body condition changes might occur, especially in males, as they have been identified as more vulnerable than females to ocean acidification.

Despite the usefulness of the biochemical endpoints analyzed, it is important in future studies to add complementary indicators, such as of DNA damage (e.g. DNA in tail, Bhagat et al., 2016) and of antioxidant properties [superoxide dismutases (SOD), peroxiredoxines, and other glutathione enzymes (e.g. including glutathione, glutathione reductase and glutathione peroxidases)] in order to reinforce the present findings. As mentioned earlier, most above-mentioned studies assessing oxidative stress only took into consideration the physiological response of the individual, ignoring potential differences inherent to sex. Our findings demonstrated that for most biochemical endpoints evaluated, the gender and temperature were the most relevant variables modelling physiological response of topshells. In the light of these findings, future biomarker studies aiming to assess environmental changes (climate or toxic-related) should optimally encompass sex as meaningful variable and rely on a multiple biomarkers approach comprising proteins, lipids and/or DNA as the oxidative damage levels can differ between different macromolecular targets (Matoo et al., 2013). Otherwise, misleading inferences remain to be considered, hampering for an accurate understanding of ecophysiological responses in aquatic and terrestrial organisms.

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